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RESEARCH PAPERS

Biological activities of green coffee nanoemulsions evaluated through alternative methods: MTT, cellular proliferation, and HET-CAM assays

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Highlights

- Nanotechnology was used to develop nanoemulsions to be tested for biological activities;
- The green coffee nanoemulsions under study did not have toxic and/or irritating effects;
- Alternative methods to animal use were used.

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KEYWORDS
Green coffee;
Cosmetic;
Nanoemulsions;
Cell viability;
Cell proliferation;
Eye irritation.

Abstract: Green coffee (*Coffea arabica*) has been widely explored by the cosmetic industry for its beneficial properties to the skin. The oil from coffee beans helps in hydration by retaining the lipids of the stratum corneum forming a barrier to the water in the skin, also preventing the aging. The green coffee paste also has positive implications in the development of cosmetic products with high biological activity, i.e., anti bacterial, antioxidant, proliferative, and aiding skin healing. This study aims to explore the synergistic effects of coffee constituents by investigating green coffee nanoemulsions. The evaluation will focus on their impact on cell proliferation, viability, as well as the potential risk of eye irritation. The findings will contribute to establishing the safety and efficacy criteria necessary for incorporating these nanoemulsions into cosmetic products. The nanoemulsions were approximately 200 nm in size, with monodispersive characteristics, and negative charged. *In vitro* biological tests showed that green coffee nanoemulsions had low cytotoxicity, stimulate cell proliferation and did not show eye irritation by the alternative methods to animal use that have been tested. The study demonstrated promising potential, but further research is necessary for its practical incorporation into the industry.

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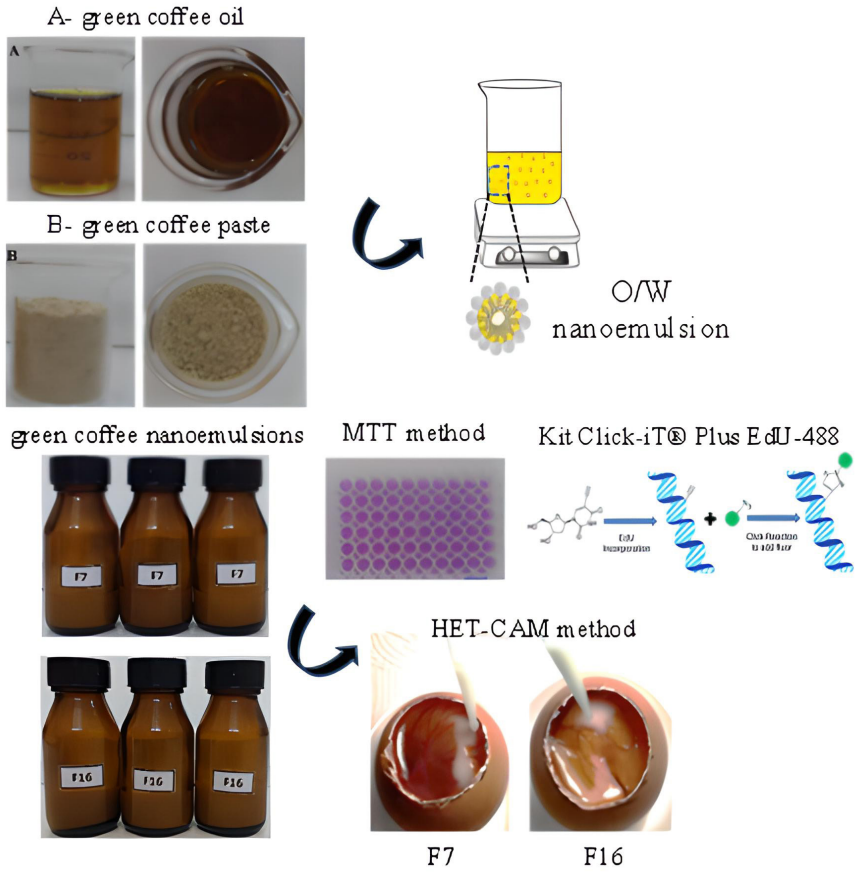
Graphical Abstract



Introduction

Skin is one of the major contributors to people's self-esteem, thus considerable attention has been given to dermocosmetic cares (Boelsma et al., 2001) that provide the maintenance of the skin barrier and/or stimulate the components present in the extracellular matrix (Stamatas et al., 2008). The topical use of cosmetics helps in the treatment of skin disorders, injured skin, as well as the prevention of photoaging and natural aging. The skin is an organ susceptible to the application of cosmetic products and consumers are increasingly demanding natural ingredients and their additives, as well as the replacement of synthetic compounds with possible negative effects on skin health (Soto et al., 2018). The structure of skin maintains a balance between the water content and the lipids present of the stratum corneum, which may be favored by the application of specific cosmetics, for example, rich in fatty acids. However, bioactive compounds added to cosmetic formulations need to overcome the skin protection barrier that may prevent the product from reaching its full biological potential (Carvalho, 2014; Carvalho et al., 2014; Harris, 2016; Abraão, 2017).

Therefore, nanoemulsion technology represents a promising approach that facilitates the penetration of cosmetics into the deeper layers of the skin, including the dermis, thereby transforming them into effective dermocosmetics. The dermis is composed of fibrous extracellular matrix with collagen and elastin, as well as by cell types such as fibroblasts that when stimulated by a dermocosmetic can minimize the natural damage caused by aging and sun exposure processes (Kanitakis, 2002; Harris, 2009; Sutradhar & Amin, 2013).

Green coffee has widely been exploited by the cosmetic industry for its beneficial properties related to the skin. The composition of green coffee oil (GCO) is about 75% triacylglycerols, 13.5% unsaponifiable matter, and 0.24% waxes (Wagemaker et al., 2012; Elizei et al., 2016). Topical application of the oil alters the mechanical characteristics of the dermal tissue, causing flexibility and hydration by retaining the stratum corneum lipids, forming a water barrier on the skin (Stamatas et al., 2008). They can also be used in anti-aging and extremely dry skin formulations. In addition, they participate in the maintenance of the skin's protective barrier, stimulate the proliferation of fibroblasts and production of components present in the extracellular matrix (Oliveira et al., 2014).

In addition to GCO, it is speculated that the residual biomass of green coffee beans following the oil fraction extraction, herein called paste, retains important bioactive compounds of interest for skin health. Thus, it is imperative more studies that investigate these residues not only at the chemical level, but also aiming at to prove eventual biological activities relevant for human health and industry (Baggio, 2006), with application in developing new cosmetic products (Puertas-Mejía et al., 2013). Besides, replacing chemical synthesized components in dermocosmetic formulations by natural compounds is conducive to the health and quality

of elaborated end products (Baggio, 2006; Andrade, 2011). Due to the synergistic effect that coffee constituents can have among themselves and because it is a natural raw material, coffee has gained strong worldwide interest as evidenced by the growing numbers of products marketed from 2011 (Koch & Parchet, 1994; Wagemaker et al., 2013).

In this context, the development of new formulations that provide greater and better interaction with the skin and its cellular constituents is necessary (Engel et al., 2008). Thus, nanocosmetic technology is an exciting emerging field with multiple promising applications in controlling the release of bioactive compounds and improving the efficience of dermal treatments (Fronza et al., 2007; Abraão, 2017). Specifically in respect to the cosmetic applications, the interest of nanoemulsions has increased due to some of their beneficial properties, such as: (1) use of low surfactant concentrations, (2) the small size of the droplets that provide uniformity in topical application, (3) greater effectiveness in delivery of the asset to the skin, increasing tissue penetration, and (4) prolonging the action time of bioactive compounds (Neves, 2008). Furthermore, the use of nanotechnology for product development is a very innovative and constantly growing area. Additionally, it is worth mentioning the adoption of alternative methods to animal experimentation in pipelines of formulations and product development for the cosmetic industry (Seaman et al., 2010).

Thus, taking into account that no previous reports of nanoemulsion systems using oil and green coffee extracts were found as proposed in this study, this work aimed to investigate the potential of green coffee nanoemulsions for cosmetic application. For that, a series of formulations were prepared and evaluated regarding their effects on cell proliferation and viability, besides the risk and/or potential to cause eye irritation, highlighting the appropriate safety and efficacy criteria that need to be fulfilled when nanoemulsions come into cosmetic products.

Material and methods

Material

Green coffee oil and green coffee paste were kindly provided by Cooxupé (Guaxupé Coffee Growers Cooperative) - Minas Gerais, Brazil. The samples of green coffee oil were obtained by cold pressing the green coffee beans (*Coffea arabica*). The residual biomass of the coffee oil extraction, hereafter refered to as green coffee paste, was collected and packed in plastic bags protected from the light and moisture, following the storage at -80°C. The chemicals used in the experiments were bought from the suppliers as follows: soy lecithin (Lipoid S75, Lipoid GmbH), poloxamer 188 (Kolliphor P188 micro, BASF), and caprylic/capric triglyceride (Miglyol 812N) provided by PIC Química (São Paulo, Brazil); ethyl alcohol (Vetec, Brazil), Dulbecco's modified culture medium (DMEM), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl bromo tetrazolium (MTT), tripan blue, sodium carbonate, sodium bicarbonate, potassium chloride, monobasic and dibasic potassium phosphate, sodium and potassium hydroxide, and formaldehyde were purchased from Vetec (Rio de Janeiro, Brazil). Phosphate buffer (PBS/Gibco, Karlsruhe, Germany), antibiotic solution containing penicillin (100 IU/mL) and streptomycin (100 µg/mL), fetal bovine serum (FBS), and trypsin EDTA were purchased from LGC Biotechnology (São Paulo, Brazil). Sodium dodecyl sulfate (SDS) and isopropanol were furnished by Sigma-Aldrich (St. Louis, MO) and Neon Comercial Ltda (São Paulo, Brazil), respectively. The kit with epidermal tissues, growth medium (SGM), and medium of maintenance (SMM) were kindly furnished by L'ORÉAL Research & Innovation Center (Rio de Janeiro, Brazil). The remaining consumables were purchased as follows: Click-iT® Plus EdU-488 cell proliferation kit (Millipore, USA), bovine albumin serum (BSA/Interlab Distr. Prod. Scientists Ltda, São Paulo, Brazil), and 4', 6'-diamino-2-phenyl-indole (DAPI/Sigma-Aldrich, St. Louis, MO).

Preparation of formulations

Firstly, the aqueous extract of green coffee beans was obtained by adding 25 mL distilled water to 2.5 g green coffee paste, following magnetic stirring for 1 h. The aqueous extract (32 mg/mL total phenolic content) was recovered by centrifuging (4000 rpm, 5 min) and stored at -80ºC. The green coffee nanoemulsions were prepared by the spontaneous emulsification method (Bouchemal et al., 2004) using different concentrations of green coffee oil, green coffee extract, soy lecithin, and hydrophilic surfactant poloxamer. The aqueous phase containing green coffee extract (v/v), water (mL), and poloxamer (w/v) was dripped in an oil phase containing green coffee oil (w/v), ethanol (v/v), and lecithin (w/v) until complete homogenization of the solution. Then, the colloidal suspensions were evaporated under pressure (700 Mpa) to a desired final volume (10 mL) to remove the organic solvent and, subsequently, filtered on cellulose membrane under vacuum. For comparison purposes, blank nanoemulsions were prepared using caprylic triglyceride (TCM) as oil under the same preparation conditions used for the green coffee nanoemulsions. All experiments were performed in triplicate. Data that are more specific cannot be provided because the product developed here is under the process of patent registration and industrial secret.

Particle size and zeta potential measurements

Dynamic light scattering (DLS) and laser-doppler anemometry using Zetasizer Nano ZS90 - Model ZEN3690 (Malvern Instruments, Worcestershire, UK) were used to determine particle size and zeta potential. This characterization was performed at 25°C after dilution (10×) of the samples in ultrapure water. The particle size was determined at a fixed angle of 90°, while the zeta potential was evaluated by placing the samples in electrophoretic cells, followed by application of an electrical potential at ± 150 mV. The zeta potential was calculated based on the average electrophoretic mobility according to the Smoluchowski equation (Smoluchowski, 1916).

pH determination

The pH of the formulations was measured in a potentiometer (Sensoglass SP1800, São Paulo, Brazil) calibrated with buffer solutions at pH 4.0 and 7.0.

Cell culture

Cell line and culture conditions

BALB/3T3 clone A31 mouse fibroblasts (*Mus musculus*) were obtained from the Bank of Cells of Rio de Janeiro (BCRJ) and cultured in DMEM culture medium supplemented with 10% (v/v) FBS (fetal bovine serum), 3.7 g/L sodium bicarbonate, 4.5 g/L D-glucose, 4 mM/L glutamine, and antibiotic combination containing penicillin (100 µL/mL) and streptomycin (100 µg/mL). Cells were cultured under humidified atmosphere, at 37°C, with 5% CO_2 supplemented.

Cell viability assay with MTT

Fibroblasts 3T3 were inoculated in DMEM culture medium supplemented with 10% FBS, in 96-well culture plates (cell density 5×10^3 cells/well, 100 µL/well). The cells were incubated for 24 h, at 37°C and 5% CO_2.

To evaluate the non-toxic nanoemulsion concentrations to the cells, a pilot test was performed at 0.1 to 3000 µg/mL nanoemulsion, for 24 h. After defining the concentrations to be used, the cells were treated with nanoemulsions ranging from 0.1 to 300 µg/mL, for 24 h. For each nanoemulsion formulation under study, a corresponding blank nanoemulsion was used.

After the time elapsed for the treatments, the culture medium was removed and an aliquot (100 µL) of fresh culture medium/well was added, plus 10 µL MTT (5 mg/mL in PBS), followed by incubation (5% CO_2, 37°C) for 3 h. Further, the medium was removed again and 100 µL DMSO (dimethylsulfoxide) were added for total solubilization of formazan crystals formed during the incubation period. The absorbance readings (540 nm) used an UV-vis spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA) and cell viability was calculated in relation to the control (100% viability).

Values were expressed as mean ± standard deviation of three individual experiments, performed in sextuplicates for each sample.

Cellular proliferation assay with Kit Click-iT® Plus EdU-488

The 3T3 fibroblasts were inoculated in six-well plates containing glass coverslips (50×10^3 cells/coverslip) previously treated with 10 µL of poly-L-lysine for better adhesion of the cells under the slides, and then incubated. After 24 h for cell adherence, green coffee nanoemulsions and blank nanoemulsion in concentrations at 0.1 to 100 µg/mL were added to the culture medium. Following treatment, 100 µL EdU (10 µM) were incorporated to the cell cultures, as specified by the manufacturer (EMD Millipore, 2015). After this period, the cells were fixed with 3.7% paraformaldehyde (200 µL/well), for 15 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 in PBS (1000 µL/well) for 20 min, followed by two washes with 3% BSA in PBS (800 µL/well). To proceed with the detection of EdU fluorescence, a reaction cocktail (100 µL/well) was prepared according to the manufacturer's instructions (EMD Millipore, 2015). The plates remained for 30 min at room temperature protected from light, and then proceeded with three washes in 3% BSA in PBS. The nuclei were marked with 4'-6-diamino-2-phenylindole (DAPI – 10 µL/well, 1: 1000, v/v) for 2 min. The fluorescence images were examined and recorded using a microscope (DMI6000 B, Leica Microsystems, GER) with 400× magnification. To quantify the number of positive cells for EdU assay, four fields/coverslip were evaluated. The proportions of the specific expression of the EdU marker were analyzed by the percentage of cells labeled in relation to the total nuclei (DAPI) in the different culture conditions analyzed (Buck et al., 2008). The images were quantified using the ImageJ 1.50i software (National Institute of Health, Bethesda, USA). The control group received only half DMEM with 10% SFB. The data were expressed as a percentage of proliferating cells of two independent experiments performed in duplicate (n=4), according to the formula:

$$\textbf{Cell proliferation}\ (\%) = (EdU \times 100\ nucleus)\ /\ DAPI\ nucleus \quad (1)$$

Eye irritation test by the HET-CAM method

The HET-CAM method followed the protocols validated by Spielmann et al. (1991) and ICCVAM (2010), taking into account the requirements of the Ethics Committee on the Use of Animals at the Federal University of Santa Catarina (protocol n° 4478120418, CEUA - UFSC). Fertile chicken eggs (*Gallus domesticus*, breed Hy Line brown) were obtained by donation (Poultry Laboratory, Department of Zootechnics and Rural Development, Federal University of Santa Catarina). The fresh and clean eggs (one day after laying, ~ 50-60g weight) were incubated for 9 days at 37.5°C in a specific incubator with a halogen lamp (temperature and humidity checked daily) to select viable eggs by ovoscopy. Eggs with cracked shells or any type of visual deformity were discarded. After confirming egg viability, the shell around the air chamber was removed with the aid of surgical scissor, the inner film was moistened with 0.9% saline solution (NaCl 0.9%) for its subsequent removal and, thus, the chorioallantoic membrane was revealed. The nanoemulsions under study were deposited directly on the chorioallantoic membrane (CAM) of the chicken egg. For that, 300 µL of each green coffee nanoemulsion were applied to the chorioallantoic membrane and the counting of the time for each event was immediately started using a stopwatch. The reactions were observed for 5 min, monitoring the emergence of endpoints such as: hyperemia (increased blood flow), hemorrhage (leakage of blood from the vessels), vascular lysis (rupture of the vessels), and coagulation (presence of blood clot) on the membrane, being noted the elapsed time (s) and the severity of the observed effect. After that, the scores for each evaluated endpoint were counted. These observations were made using a magnifying glass with a 0.65-0.80× magnification (Stemi 2000-C - Carl Zeiss, FRANCE).

The irritation score (IS), determined by the time (s) in which each event was recorded, was calculated using the formula below, with the values being compared to the categories of irritation (Spielmann et al., 1991). The IS values resulted from the average of three independent evaluations (3 eggs) for each nanoemulsion under test, with repetition of three experiments for each treatment (n = 9).

$$\begin{aligned}(\mathbf{IS}) = \{&(301 - bleeding\ time/300) \times 5 + \\ &(301 - lysis\ time/300) \times 7 + \\ &(301 - coagulation\ time/300)\end{aligned} \quad (2)$$

As a negative control, a 300 µL NaCl 0.9% solution was used in order to provide a baseline for the end points of

the assay. As a positive control, 300 µL 1% SDS solution, an irritant known to induce severe response *in vivo* and in the HET-CAM method were used.

The validated HET-CAM protocols consider that the 5 min exposure period of the chorioallantoic membrane to the test substance is sufficient to cause irritating/toxic effects, and longer time exposures did not result in any additional information.

After the completion of each experiment, the eggs were transferred to a freezer (-20°C, for 12 h) to euthanize the embryos without causing pain. According to the Institutional Committee for the Care and Use of Animals (IACUC), embryos until 10 embryonic days are said to be incapable of feeling pain, but after their experimental usage, they must be sacrificed by hypothermia (Institutional Animal Care and Use Committee, 2016).

Statistical analysis

The data were collected and summarized, following statistical analysis of variance (ANOVA) and when appropriated the multiple comparisons Tukey and Dunnett's post-hoc tests were applied with the aid of Graph-Pad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). P values < 0.01 were considered significant.

Results

In this work, two nanoemulsified green coffee systems were developed by the spontaneous emulsification method. The nanoemulsions F7 and F16 (Figure 1) presented a milky appearance and slightly yellow color, while the blank one (NC- without the bioactive compounds) showed a white coloration.

The particle size of the F7 and F16 nanoemulsions was around 200 nm according to the method of dynamic light scattering, as shown in Figure 2.

It can be noted that with the increase in the concentration of coffee oil from 0.1 g/mL (F7 nanoemulsion) to 0.25 g/mL in the F16 nanoemulsion, an increase in the size of the nanoparticles occurred. The formulations presented monodisperse distribution (PdI <0.25) with zeta potential values ranging from -42.2 to -48.2 for F7 and F16, respectively. Regarding the pH, the F7 and F16 nanoemulsions showed value of 6.15 and 6.10, respectively.

In a second approach to investigate the biological activity of the nanoemulsions under study, experiments were carried out with BALB/c 3T3 fibroblasts cell lines, clone a31, in order to verify eventual cytotoxicity and eye irritation effects, as well as to measure the cell proliferation following exposure to the green coffee nanoemulsions.

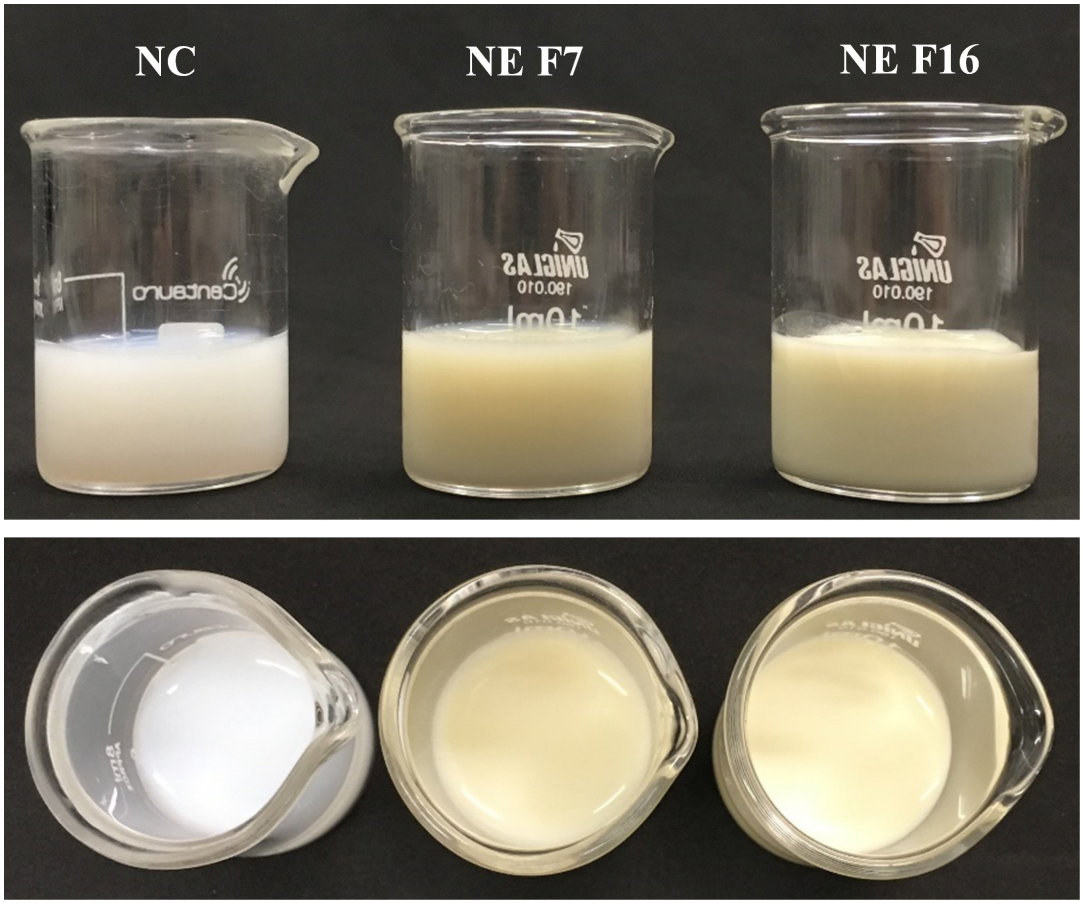


Figure 1. Physical appearance of the nanoemulsions obtained by the spontaneous emulsification method. Blank nanoemulsion (NC - without the bioactive compounds); nanoemulsion F7 (NE F7) and nanoemulsion F16 (NE F16).

The results obtained in the MTT assay are presented as percentage of cell viability as a function of the concentrations of green coffee nanoemulsions investigated (Figure 3).

The F7 formulation exhibited non-toxicity towards cells up to a concentration of 100 µg/mL, as evidenced by cell viability values exceeding 50% (Figure 3). However, higher nanoemulsion concentrations, i.e., 300 and 1000 µg/mL, showed to be toxic, differing statistically from all other concentrations tested. Additionally, it is evident that all F7 nanoemulsions treatments showed differences compared to control.

Regarding the formulation F16, the treatments differed statistically in their cytotoxic effects in respecto to control. A cause/effect relation between nanoemulsion concentrations and citotoxicity was not clearly observed, since at 30 and 100 µg/mL nanoemulsions were toxic to fibroblasts, while higher values increased the cell viability. The reasons for the decrease in cellular viability are not known; however, since it is a first experiment, subsidies regarding the doses to be used in the next experiments were obtained.

In a second-round experiment, F7 nanoemulsions at 300 and 1000 µg/mL were excluded, because their cytotoxic effects as previously shown. The formulation F7 proved to be non-toxic up to a concentration of 100 µg/mL (Figure 4), besides increasing cellular viability between 115% to 125% in relation to control (100%), suggesting evidence of cell proliferation. In addition, this formulation also proved to be more effective in promoting cell proliferation than the blank nanoemulsion up to a concentration of 100 µg/mL.

The F7 nanoemulsion at 0.1 to 30 µg/mL presented a stimulating effect on cell viability, as those concentrations differed from the control and the blank nanoemulsion ($p < 0.05$), except for the treatment with F7 at 30 µg/mL, which did not differ from the blank nano (Figure 4). Besides, F7 nanoemulsion at 100 µg/mL was superior in cell viability compared to the blank one and similar to control.

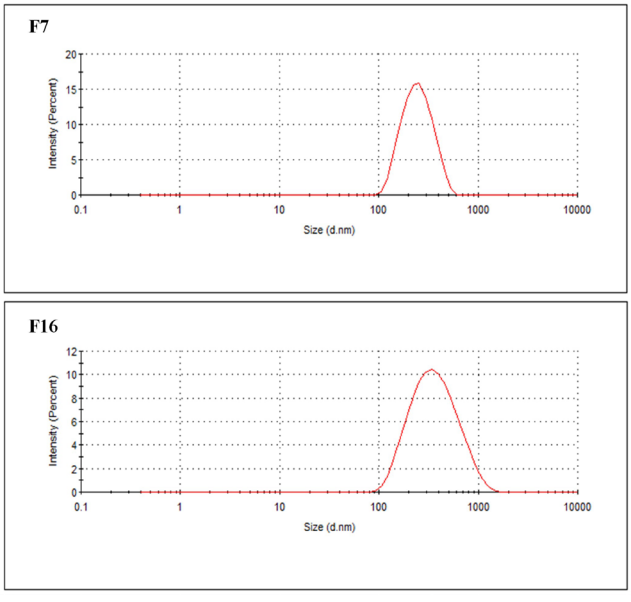


Figure 2. Particle size distribution of the F7 and F16 green coffee nanoemulsions obtained by the dynamic light scattering method.

Figure 5 reveals no significant differences between formulation F16 and blank nanoemulsion on cell viability. When compared to control, both nanoemulsions at 100 µg/mL reduced cell viability, however, still with values above 50% viability.

The analysis of the results of the two nanoemulsions and their controls (blank ones) revealed greater cell viability for the F7 formulation compared to the F16 one and controls.

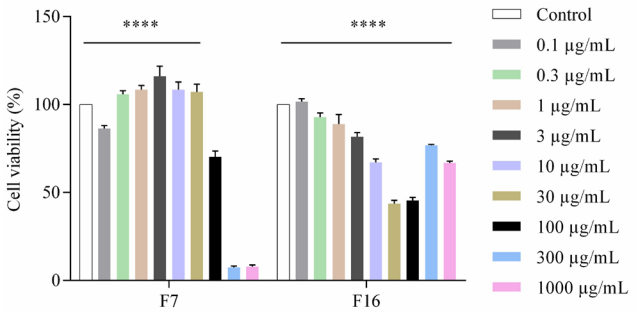


Figure 3. Cell viability (%) determined by the MTT assay in 3T3 fibroblasts after incubation for 24 h with F7 and F16 green coffee nanoemulsions (0.1 - 1000 µg/mL). **** $p < 0.0001$ (ANOVA followed by Tukey's and Dunnett's test).

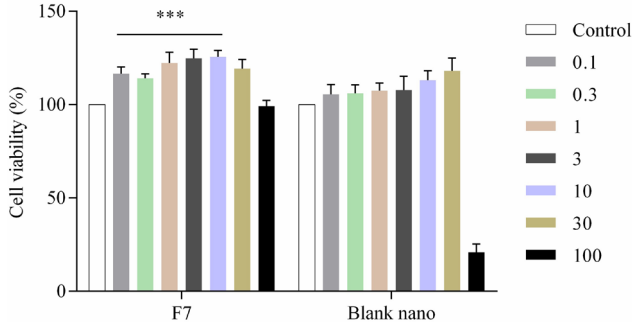


Figure 4. Cell viability (%) determined by the MTT assay in 3T3 fibroblasts cell lines, after incubation for 24 h with F7 and blank nanoemulsions (0.1 - 100 µg/mL). *** $p < 0.001$ (ANOVA followed by Tukey's and Dunnett's test).

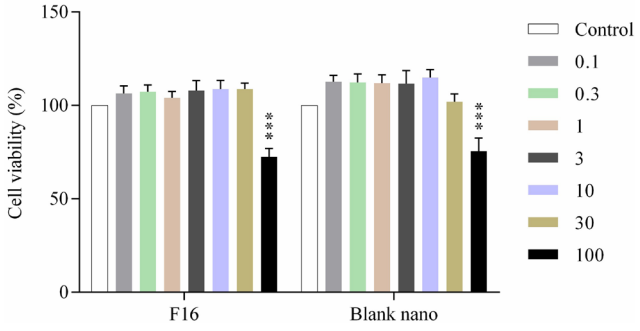


Figure 5. Cell viability (%) determined by the MTT assay in 3T3 fibroblasts cell lines, after incubation for 24h with F16 (0.1 - 100 µg/mL) and blank nanoemulsions. *** $p < 0.001$ (ANOVA followed by Tukey's and Dunnett's test).

However, none of the nanoemulsions investigated proved to be toxic or irritating to the 3T3 fibroblasts cell lines.

As shown in Figure 6, nanoemulsions were evaluated for their proliferative effect in 3T3 fibroblasts cell cultures.

In Figure 6, its can be seen that F7 nanoemulsion induced a cell proliferative effect as of 1 µg/mL, reaching the greatest stimulus at 10 µg/mL with 88% cell proliferation, being superior to its control (65.1%, p <0.0001). Regarding the F16 nanoemulsion, a similar result was observed, with increased cell proliferation by up to 75.2% (10 µg/mL); also higher than the control (50.6% - p <0.0001). The blank nanoemulsion (negative control) showed cell proliferation values of 73.4% in the same concentration (10 µg/mL), lower than the control (80.6%).

In addition, it is possible to detect an eventual dose-effect relation regarding the induction to the cell proliferation in the 0.1 – 10 µg/mL concentration range for both F7 and F16 nanoemulsions. On the other hand, nanoemulsions concentrations higher than 100 µg/mL showed cellular toxicity, as demonstrated by the results of the MTT assay (Figure 3).

The results concerning the eye irritation potential assay of nanoemulsions and controls by the *in vivo* method of HET-CAM are shown in Table 1.

No significant differences were detected between treatments with F7 and F16 nanoemulsions and the negative control in eye irritation (Figure 7). The F7 nanoemulsion caused only one event (n = 9) where mildly eye irritation was detected, with a score of 1.29 resulting from the bleeding effect. This may have occurred due to an eventual damage caused to the CAM during manipulation, which was not visually detected. Therefore, according to the HET-CAM assay findings, both F7 (Figure 7C) and F16 (Figure 7D) nanoemulsions were not irritating to CAM and neither toxic, being considered equivalent to what was presented by the

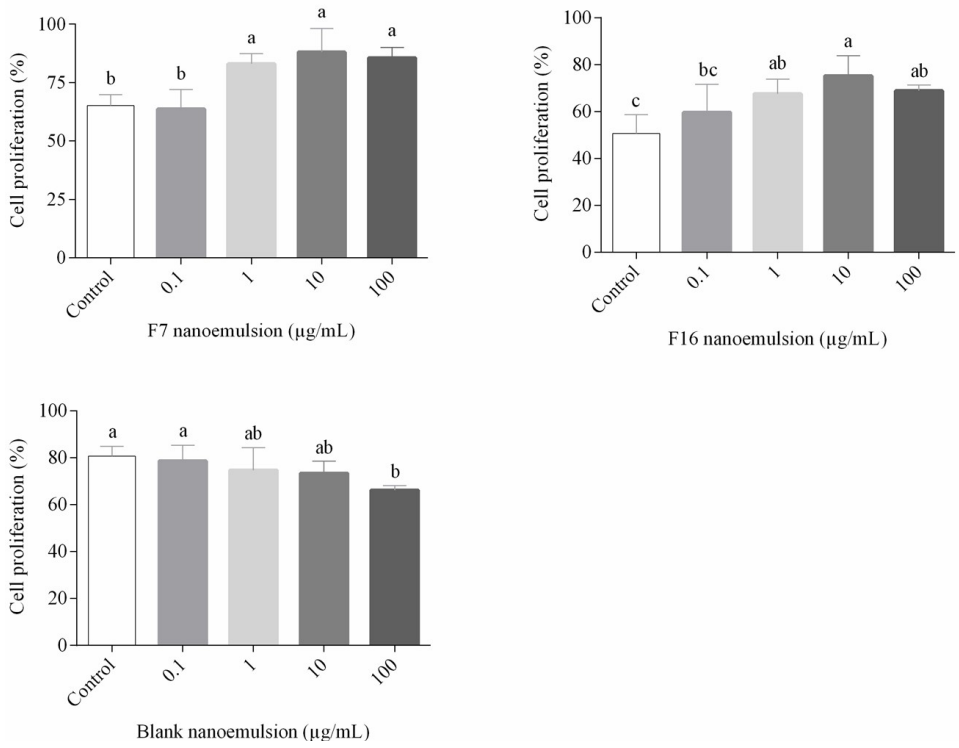


Figure 6. Effect of green coffee nanoemulsions (0.1 - 100 µg/mL) on cell proliferation of 3T3 fibroblasts, after 24 h of exposure determined by the Click-iT® Plus EdU-488 Kit. Different letters (a, b, c) indicate statistical differences (p <0.0001) between treatments and control (Tukey test).

Table 1. Irritation score, category of irritation, and effect classification of F7 and F16 nanoemulsions, SDS (1% w/v) and NaCl (0.9% w/v), according to the HET-CAM assay.

Compound	Irritation score*	Category of irritation	Classification of effect
Nanoemulsion F7	1.29 ± 0.48	1 - 4.9	Slightly irritating
Nanoemulsion F16	0.413 ± 0.18	0 - 0.9	Non-irritating
SDS 1% (positive control)	16.88 ± 0.42	9 - 21.0	Severely irritating
NaCl 0.9% (negative control)	0.07 ± 0.00	0 - 0.9	Non-irritating

*p <0.001 according to the Tukey's test. Data are represented as mean ± standard deviation (SD) of three independent replicates (n = 9).

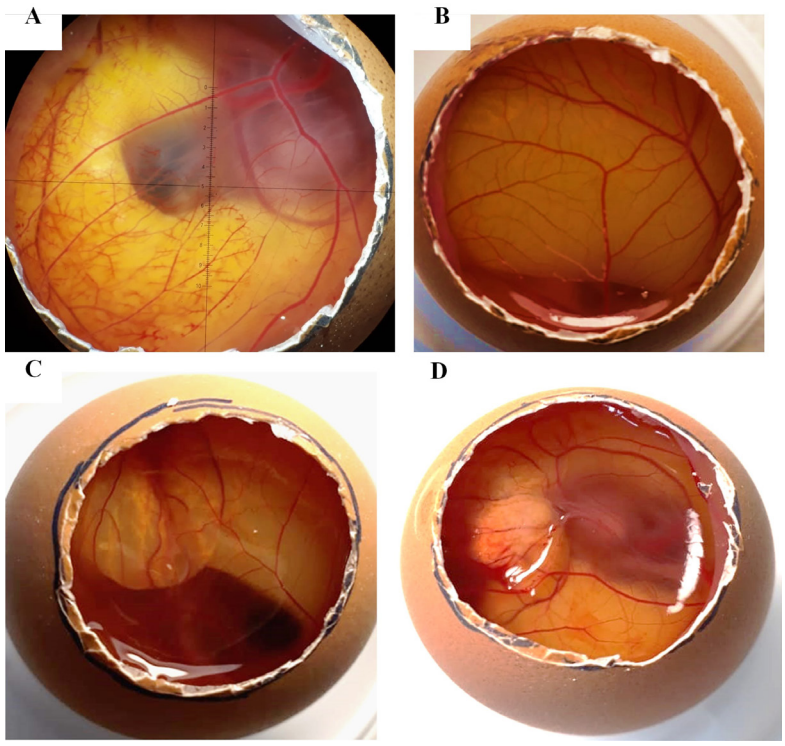


Figure 7. *In vivo* HET-CAM assay: CAM treated with SDS 1% (A), 0.9% NaCl (B), F7 nanoemulsion (C), and F16 nanoemulsion (D). The effect of F7 and F16 nanoemulsions and controls on the CAM's surface irritation was recorded by a photographic câmera, 5 min after application of the treatments.

negative control (Figure 7B). Indeed, the green coffee nanoemulsions investigated permeated the CAM and did not provide any apparent irritation or damage to the blood vessels. Contrarily, the positive control (SDS 1%) induced great damage to the CAM (Figure 7A), as noted by the enlargement of blood vessels and the appearance of small vessels with multiple branches, hemorrhage, and blood coagulation.

Discussion

Considering the different methods for the preparation of nanoemulsified systems, i.e., high and low pressure, the former one has a high cost. Therefore, there is a need to explore techniques for the development of nanoemulsions that are economical and fast, minimizing processes, especially considering large-scale industrial applications (Bajerski et al., 2016). The spontaneous emulsification method lowers production costs, where the constituents of an oil phase drips into an aqueous phase, providing an oil-water interface, which when subjected to magnetic stirring increases the surface tension between the two phases, resulting in the formation of nano-drops. This was the method used in the preparation of green coffee nanoemulsions (Gupta et al., 2016; Jintapattanakit, 2018).

Nanotechnology seeks to optimize the penetration of actives into the skin, enhancing its moisturizing properties and protection against external damage (Beck et al., 2011). According to the particle size (≈ 200 nm) of the F7 and F16 nanoemulsions, it can be inferred that these green coffee formulations can penetrate the skin layer, exerting their effect on the dermis.

Polydispersion indices below 0.25, as observed for the green coffee nanoemulsions investigated, indicate adequate homogeneity of nanoemulsified systems (Almeida et al., 2009), while high zeta potential values, in modulus, are essential to provide physical stability to the nanoemulsion system. The nanoparticle's negative surface potential is due to the negative charge of the lecithin surfactant used (Mazzarino et al., 2017) in the formulations. In addition to these variables, the pH of the formulation is also considered important, as it influences the particle size and consequently the stability of the colloidal system (Addor & Silva, 2013; Nirmala & Nagarajan, 2017). Importantly, the pH of the green coffee nanoemulsions (≈ 6.15) is applicable to topical use, in accordance with the pH range of a healthy skin (between 5.5 and 6.5), that is, an environment slightly acidic (Maruno, 2009).

According to Maruno (2009), nanoemulsions containing particles with very small size (20 - 500 nm) have as one of their main characteristics to prevent the process of physical instability of the formulation. The author produced two nanoemulsions containing castor and raspberry oils to treat skin burns caused by excessive sun exposure. The nanoemulsions were not satisfactory in preventing burns, but they accelerated the reduction of the erythematous area.

According to Maciel (2012), the development of multiple emulsions containing sunflower oil and sesame oil and the use of low concentrations of surfactants is essential when

it comes to the production of nanoemulsions for cosmetics, as surfactants in large quantities can cause skin irritations/allergies. When it comes to the active ingredient, the incorporation of a combination of two or more compounds, as commonly observed in formulations containing essential oils, is regarded as beneficial. This is because there is a potential for synergistic interactions among the bioactives, leading to enhanced desired effects of the formulation. In this sense, it is worth emphasizing the importance of the nanoemulsions developed in this work, as they make use of low concentrations of surfactants, about 0.02 g/mL of the formulation. In addition, two biomasses (green coffee extract and oil) have been used, aiming at the synergistic effect between bioactive compounds of those chemically complex matrices, potentiating the eventual biological/pharmaceutical activities expected by the formulations. In addition, the developed technology makes use of coffee industry waste, an environmental problem in Brazil and, therefore, the use of these biomasses in the investigated product assumes importance in the economic and environmental spheres.

Cytotoxicity tests are necessary whether one aims at to determine the harmful potential to cell metabolism and viability of raw materials, for example, when developing new products of biotechnological interest (Aslantürk, 2018). Therefore, green coffee nanoemulsions F7 and F16 were analyzed by the MTT assay to determine their cytotoxic potential. This method was chosen because it is a relatively simple test, validated by regulatory agencies, and widely used for toxicity tests (Organisation for Economic Co-operation and Development, 2014). According to the regulatory guides of the Organisation for Economic Co-operation and Development (2014), test substances are considered toxic/irritating when they reduce cell viability below 50%. Conversely, those with viability above 50% are designated as non-toxic.

Green coffee nanoemulsions, according to OECD recommendations (2014), did not show toxicity/irritation to fibroblasts under the conditions evaluated in this study. In addition, there was good cell's tolerance following exposure to the green coffee nanoemulsions in the range of concentrations evaluated, which suggests that the investigated formulations can be used safely for topical purposes.

According to Gasque et al. (2014), cell density (i.e., inoculum density) is a relevant aspect in viability tests, as the cell's responses to the toxicity of a given compound are directly related to that variable. Although the literature states that lower cell densities suffer greater action when exposed to test substances, such behavior was not observed in the experiments. On the other hand, the percentage of cell viability was higher in all treatments, with cell densities of 2.5×10^3 cells/well. According to the literature, a lower cell density is advantageous for strains with a rapid proliferation rate like the 3T3 fibroblasts cell line used in this study (Gasque et al., 2014). In fact, cell cultures would not reach confluence before the end of the experiments, preventing damage to the cells or even their death during the tests.

According to Aslantürk (2018), to establish the degree of safety of a product, first tests of cell viability should be performed *in vitro*. Even in the case of natural ingredients, research on the safety of a given compound is essential. When cytotoxicity is considered absent, or irrelevant, one can proceed with subsequent tests to prove the product's effectiveness, as well as *in vivo* tests (Aslantürk, 2018). Therefore, this was the reasoning used in this work, being that the cytotoxicity studies described for green coffee nanoemulsions are considered as a first step in the process of developing products formulated with compounds from that biomass, serving as a guide for other tests with similar nanoderivatives.

During the aging process, biomechanical changes in the skin occur, which are mainly caused by the ability of decreased cell proliferation and collagen degradation, reflecting changes in the extracellular matrix of the dermis. However, these processes can be mitigated or reversed to some extent with the use of bioactive compounds (Harris, 2016). Thus, studies that investigate and develop appropriate topical formulations are essential to alleviate this problem. Therefore, to investigate the inducing effect of green coffee namoemulsions on cell proliferation, the Click-iT® Plus Kit EdU-488 was used. For that, 3T3 fibroblasts cell cultures were grown in the presence of the reagent 5-ethinyl-2'-deoxyuridine (EdU), allowing thymidine bases to be incorporated into nascent DNA during the cell synthesis phase (phase S), being subsequently detected and quantified by fluorescence microscopy.

According to Afornali et al. (2013), the results presented allow us to infer that the investigated nanostructured systems significantly increased the biological effects investigated due to the nanoencapsulation of green coffee oil. This fact can be confirmed through comparative analysis with the results of Voytena (2017), who used free green coffee oil, that is, not encapsulated, and found lower values of cell proliferation. Thus, it is assumed that the higher surface-volume ratio of the nanoparticles improved the cell absorption of the bioactive compounds. Therefore, the use of nanoemulsions containing a diversity of bioactive polar and apolar compounds (oil + green coffee extract) appears as an interesting possibility for the treatment and prevention of skin-related problems.

After cytotoxicity and proliferation investigations with the cell line presented, it was decided to use another alternative method, now with the objective of analyzing the irritation potential in face of green coffee nanoemulsions. The HET-CAM test has the ability to assess the irritating potential of formulations containing surfactants, as in the case of the nanoemulsions herein studied, having a good correlation with the concentration of the investigated product (Zanatta, 2008; Maciel, 2012). In addition, CAM has a vascularized structure similar to the human conjunctiva, being, therefore, important in the response to irritating formulations and in the appropriate choice of compounds for the preparation of nanoemulsions for topical purposes (Leite, 2018). Several tests can be used to assess eye irritation from test substances: bovine corneal opacity and permeability test, isolated rabbit eye test, isolated chicken eye test, and test of the chicken egg (HET-CAM). In this study, we adopted the HET-CAM assay, as the literature states that this method is easily correlated with cytotoxicity tests, in addition to minimizing false positive and negative results (Bouranen, 2017). It has also been considered as an alternative to animal assay, since the chorioallantoic membrane that surrounds the developing embryo is highly vascularized and embryos in the stage of development used in the test are not sensitive to pain (Mendoza & Saavedra,

2013). The HET-CAM method allows the evaluation of the eye irritation potential of test substances, as they induce toxicity in the chorioallantoic membrane (CAM) in chicken eggs (*Gallus domesticus*). This test allows identifying possible occurrences of hemorrhage, vascular lysis, and blooding clotting. Thus, the level of irritation of the substance of interest can be classified according to the damage (or absence of) to the mucous membranes (particularly in the heart) *in vivo*. The evaluations are carried out individually and in a combined manner, making it possible to determine a score, which is used to classify the irritation potential of test substances after 5 min exposure. Additionally, the HET-CAM method is suitable for screening natural products with regard to their anti-inflammatory activity and cytotoxic potential of complex matrices or even isolated compounds (Mendoza & Saavedra, 2013). To date, there are no reports of studies investigating eye irritation by the HET-CAM method in green coffee biomasses.

For the assay to be considered acceptable, the negative (NaCl 0.9%, w/v) and positve (SDS 1%, w/v) controls must induce a non-irritating and severely irritating rating response, respectively (Figure 7A and 7B). Reports from the HET-CAM method shown that by using 0.9% NaCl the irritation score (IS) value is close to zero, while for SDS 1% values between 9 and 21 are observed for that variable (ICCVAM, 2010), as reported for the nanoemulsions of interest (Table 1). Thus, it can be inferred that the formulations developed do not have the potential for eye irritation and can be applied to the dermis.

Finally, no data were found in the literature testing green coffee nanoemulsions adopting alternative methods to animal experimentation, not even nanoderivatives containing the bioactive compounds of interest (extract and green coffee oil). Therefore, these investigations reflect the innovative character of the work presented, putting this study in evidence.

Conclusion

It can be concluded that green coffee nanoemulsions have been optimized and successfully prepared by the spontaneous emulsification method, presenting nanometric sizes suitable for skin applications, in addition to important variables for maintaining a stable colloidal system. As to the product safety, cytotoxicity tests allowed us to infer that F7 and F16 nanoemulsions were not toxic, according to the MTT test. In addition, the synergism of bioactive compounds presente in green coffee extract and oil eventually provided greater proliferative stimulation to the cell cultures. The nanostructured system formulated also showed promissing for topical application, as they were not irritating to eyes as herein shown. In addition, the nanoformulations developed by using coffee industry waste have a natural and an environmental appeal, also because they have been evaluated in accordance with European legislation using alternative methods to animal testing. Thus, the green cofee nanoemulsions have been considered promising products for use in the pharmaceutical and cosmetic industries.

Conflict of interests

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Erratum

ERRATUM: Biological activities of green coffee nanoemulsions evaluated through alternative methods: MTT, cellular proliferation, and HET-CAM assays

Due to desktop publishing error the article “Biological activities of green coffee nanoemulsions evaluated through alternative methods: MTT, cellular proliferation, and HET-CAM assays” (DOI https://doi.org/10.4322/biori.00112023), published in Biotechnology Research and Innovation, 2023, 7(1): e2023011, was published with an error that caused an author’s name to be incorrect.

On page 1, where the name reads:
Ana Paula Voyten[a,b]

It should read:
Ana Paula Voytena[b]